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EFFECT OF TWO WHEAT CULTIVARS DIFFERING IN HYDROXAMIC ACID CONCENTRATION ON DETOXIFICATION METABOLISM IN THE APHID *Sitobion avenae*

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Abstract—Hydroxamic acids (Hx) are wheat secondary metabolites conferring resistance for cereals against aphids. The activity of five enzymatic systems were evaluated in the aphid *Sitobion avenae* reared on the high-Hx wheat cultivar Chagual and the low-Hx wheat cultivar Huayún for 10 generations. Enzyme solutions were prepared from aphid homogenates and assayed for mixed function oxidases (including cytochrome *P*-450 monooxygenases and NADPH cytochrome *c* reductase), glutathione *S*-transferases, esterases, and catalase. Specific activities per aphid individual of cytochrome *P*-450 monooxygenases, NADPH cytochrome *c* reductase, glutathione *S*-transferases, and esterases were significantly increased in wheat cultivars relative to oat (only marginal increase of esterases in Chagual). Aphids fed on cv. Huayún showed an overall higher induction of enzymatic systems than those fed on cv. Chagual. Comparison of these results with reported effects of Hx on detoxifying enzymes in other insects, including aphids, support the hypothesis that these enzymatic pathways play an important role in the detoxification of toxic host-plant secondary metabolites.

Key Words—*Sitobion avenae*, aphids, hydroxamic acids, DIMBOA, detoxification, cytochrome *P*-450 monooxygenases, NADPH cytochrome *c* reductase, glutathione *S*-transferases, esterases, catalase.

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INTRODUCTION

Aphids (Hemiptera: Aphididae) are important pests of major cereal crops in temperate countries. These insects affect grain production by disrupting plant tissues, consuming essential nutrients, transmitting viruses, and providing a medium for fungal development (Minks and Harrewijn, 1987).

Plant secondary metabolites confer resistance against herbivorous insects (Rosenthal and Berenbaum, 1991). One of the main groups of secondary metabolites involved in cereal resistance to aphids are hydroxamic acid (Hx) (Niemeyer, 1988; Thackray et al., 1990; Niemeyer and Pérez, 1995). The most abundant of these acids in wheat extracts is 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) produced by hydrolysis of the naturally occurring glucoside by β-glucosidases released upon tissue injury (Hofman and Hofmanova, 1969) (Figure 1). Studies performed on cereal aphids reared on different wheat cultivars have shown that decreased performance and reproduction are related to the presence of Hx in variable concentrations (Argandoña et al., 1982; Leszczynski and Dixon, 1990; Givovich and Niemeyer, 1995). Thus, negative relationships between the intrinsic rate of increase (r_m) (Bohidar et al., 1986; Thackray et al., 1990), mean relative growth rate (MRGR) (Thackray et al., 1990), and feeding deterrency (Niemeyer et al., 1989) for the aphid *Sitobion avenae* (Fabricius) and Hx concentration in wheat plants have been described.

Cereal aphids exposed to Hx-containing hosts contain Hx (Niemeyer et al., 1989), and they detoxify the compounds through enzymatic mechanisms (Leszczynski et al., 1994). Few studies have explored the association between the activity of detoxifying enzymes in aphids and Hx in their host plants (Leszczynski and Dixon, 1992; Leszczynski et al., 1994; Figueroa et al., 1999). Three major detoxification enzymatic systems are considered important pathways for allelochemical metabolism in insects: mixed function oxidases (MFO), including cytochrome *P*-450 monooxygenases (Cy*P*450), NADPH cytochrome *c* reductase (NADPH cyt red), glutathione *S*-transferases (GSTs), and esterases (Yan et al., 1995). Furthermore, oxidative metabolism may yield free radicals (Ahmad et al., 1986), in which case catalase, whose activity is induced by the presence of oxygen free radicals, may be considered an additional pathway for detoxification of plant allelochemicals.

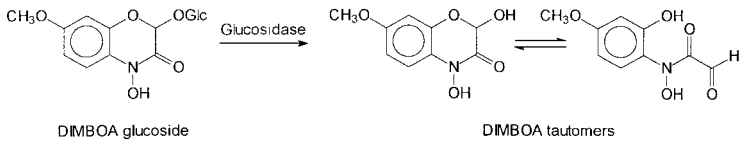

FIG. 1. Hydroxamic acids involved in aphid–cereal interactions.

The structural features of Hx (Figure 1) suggest that they are potential substrates for the enzyme systems mentioned above. Thus, lipophilicity, an electron-rich aromatic ring, and a methoxy substituent may allow hydroxylation and dealkylation reactions catalyzed by mixed function oxidases; and the electrophilic carbonylic moieties, particularly in the open tautomer of DIMBOA (Pérez and Niemeyer, 1989), and the hydroxamic nitrogen atom, which may act as an electrophile (Hashimoto and Shudo, 1996), are potential sites for conjugation with glutathion catalyzed by GSTs and/or for reaction with active nucleophilic residues of esterases.

We hypothesize that the enzymes involved in the detoxification metabolism of the aphid *Sitobion avenae* are affected by the concentration of Hx in the host plants on which they feed. In order to evaluate the effect of Hx on the activity of these enzymes, aphids were reared on two different wheat cultivars and on oat, a cereal lacking Hx, for 10 aphid generations, a period comparable to that spent on wheat fields in a season. Comparisons between wheat and oat treatments allowed the evaluation of the magnitude of Hx effects on enzymatic activity.

METHODS AND MATERIALS

Chemicals. 1-Chloro-2,4-dinitrobenzene (CDNB), α-naphthyl acetate (α-NA), fast blue B salt (FBB), *p*-nitroanisole (*p*NA), and *p*-nitrophenol (*p*NP) were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). α-Naphthol was from Merck (Darmstadt, Germany).

Insects. A multiclonal stock culture was maintained for several generations on oat (*Avena sativa* L., cv. Nehuén) in a greenhouse under conditions (22 ± 2°C, 14L : 10D photoperiod) that insure aphid reproduction is parthenogenetic.

Plants. Experiments were performed with seedlings of oat (*A. sativa* cv. Nehuén), and of two wheat cultivars differing in their Hx concentration: low-Hx (*Triticum aestivum* L.) cv. Huayún, and high-Hx (*T. durum* L.) cv. Chagual (mean ± SE: 0.667 ± 0.097 and 3.595 ± 0.257 mmol/kg fresh wt, respectively, $N = 10$, two-leaf seedlings). Seeds of oat and wheat were provided by the Instituto Nacional de Investigaciones Agropecuarias (INIA-La Platina, Chile).

The Experiment. In order to obtain synchronized aphid individuals to start the experiment, ca. 800 adult aphids were separated from the colony and placed on 8-day-old oat seedlings growing in 14.5-cm-diam. pots containing sterilized compost (H. Lee M.). The aphids were left overnight (12 hr) to reproduce and were removed the next day. Two hundred fifty synchronized first-instars from the progeny were then placed on 8-day-old oat seedlings in each of three pots and kept for nine days until they reached the adult reproductive stage (Dixon, 1998). Thus, synchronized adult apterous aphids were obtained at the end of this period and used for the experiments with different plant conditions. Groups of 200 syn-

chronized adult aphids were then placed on two-leaf seedlings of either oat (an Hx-lacking cereal used as a control) (Niemeyer, 1988), wheat cv. Chagual, or wheat cv. Huayún. These groups were considered as the first generation (GI) of the experiment. Aphids were allowed to reproduce for six days. Progeny were removed and placed on new seedlings and left for five days, by which time aphids had attained adulthood. Two hundred of these aphids were then placed on new seedlings, initiating the second generation (GII). The remaining adult aphids were counted and collected in 1.5-ml Eppendorf tubes for enzyme assays. This procedure was repeated for the next eight generations for each plant condition. Each generation lasted 11 days. Aphids from the stock culture reared on oat were considered as generation zero (G0) of the experiment.

Preparation of Subcellular Fractions for Enzyme Assays. Between 150 and 600 wingless adult aphids collected in 1.5-ml Eppendorf tubes were killed by freezing at −20°C for 30 min. Aphids were homogenized in 400 μl ice-cold homogenization buffer (10 mM Tris HCl, 1 mM EDTA, 0.2 mM DTT, 1 mM solution azide, pH 7.8) with a plastic pellet pestle (Kontes) in ice (Figueroa et al., 1999). The homogenate was centrifuged at 10,000*g* for 10 min at 4°C in an IEC Micromax RF refrigerated microcentrifuge to obtain a pellel containing nucleus and mitochondria and a supernatant containing the rest of the membranous organelles and the cytosol, according to conditions established previously (S. Mukanganyama, personal communication). This fraction was centrifuged at 105,000*g* for 40 min at 4°C in a Ti50 rotor in a Beckman L5-50 ultracentrifuge to obtain an organellar pellet (P fraction mainly containing peroxisomes, lisosomes, and microsomes), and a supernatant (S fraction) (Santos et al., 1988; Figueroa et al., 1999). The P fraction was gently resuspended in 450 μl ice-cold TGE buffer (50 mM Tris HCl, 1 mM EDTA, and 20% glycerol, pH 7.4) (S. Mukanganyama, personal communication).

Enzyme Assays. Assays for five enzymes were conducted: cytochrome *P*-450 monooxygenases (Cy*P*450), NADPH cytochrome *c* reductase (NADPH cyt red), glutathione *S*-transferases (GSTs), esterases, and catalase. Cytochrome *P*-450 monooxygenases, GSTs, and esterases assays were previously optimized with respect to enzyme concentration and incubation time. Each enzyme assay was replicated four times. Protein concentrations were determined by the Bradford method (Bradford, 1976), with BSA as standard. Absorbances for Cy*P*450, NADPH cyt red, GSTs, and esterases assays were recorded from a Shimadzu UV-240 spectrometer. Absorbances for catalase were recorded from a Packard Spectra Count spectrometer.

Cytochrome *P*-450 monooxygenase *O*-demethylase activity was determined in the P fraction with *p*-nitroanisole (*p*NA) as substrate (Kanga et al., 1997). Absorbance was measured at 400 nm against a blank lacking the enzyme, and converted to concentration of *p*NP based on a standard curve.

NADPH cytochrome *c* reductase activity was determined as previously

described (Santos et al., 1988; Figueroa et al., 1999) with the P fraction and NADPH as substrate. Increasing absorbance was registered at 550 nm in 30-sec intervals over 5 min at 25°C. Specific activity was determined from an extinction coefficient of 19.6 mM^{-1} cm^{-1} for cytochrome *c*.

Glutathione *S*-transferase activity was determined with the S fraction. Activity was determined as the conjugation of CDNB with reduced glutathione according to the modified method of Yan et al. (1995). Absorbance was measured at 340 nm against a blank lacking the enzyme. Specific activity was calculated based on an extinction coefficient of 9.6 mM^{-1} cm^{-1} for the conjugate *S*-(2,4-dinitrophenyl)-glutathione (DNPG) (Leszczynski and Dixon, 1992).

General esterase activity was determined in the S fraction according to Ortego et al. (1998). Absorbance was measured at 600 nm against a blank lacking the enzyme, and converted to concentration of α-naphthol based on a standard curve.

Catalase activity was determined in the P fraction as previously described (Santos et al., 1988; Figueroa et al., 1999). The reaction was performed at 0°C, and the absorbances measured at 405 nm.

In order to assess the effects of detoxification metabolism in the average individual, all specific activities were divided by the total number of aphids present in the homogenates to obtain the average activity per individual.

Statistical Analysis. In order to compare the effects of wheat cultivars on the rate of change of enzymatic activity over ten aphid generations, an ANCOVA was performed with plant species/cultivar as the main factor, specific activity as the dependent variable, and aphid generation as the covariate.

RESULTS AND DISCUSSION

The activity of five enzymes involved in the detoxification of xenobiotics was determined in extracts of aphids reared for 10 generations in two wheat cultivars (one with a low and the other with a high concentration of hydroxamic acids) and one oat cultivar. This latter was used as a control since isogenic wheat lines lacking Hx are not available. In order to evaluate the rate of change of enzymic activity along the generations, the data were fitted to simple linear models (Figure 2), although it showed considerable fluctuations in some cases. The slopes of the lines generated were compared by ANCOVA. An overall trend for increased specific activity per individual aphid along the generations was observed in four of the five enzymes studied in aphids from wheat (Figure 2A–D). The values of the slopes and the *P* values for the statistical significance between different host plants are shown in Table 1. The *P* values for the comparisons between the slopes of oat with a line of slope zero were 0.71, 0.852, 0.001, 0.37, and 0.128, for Cy*P*450, NADPH cyt red, GSTs, esterases,

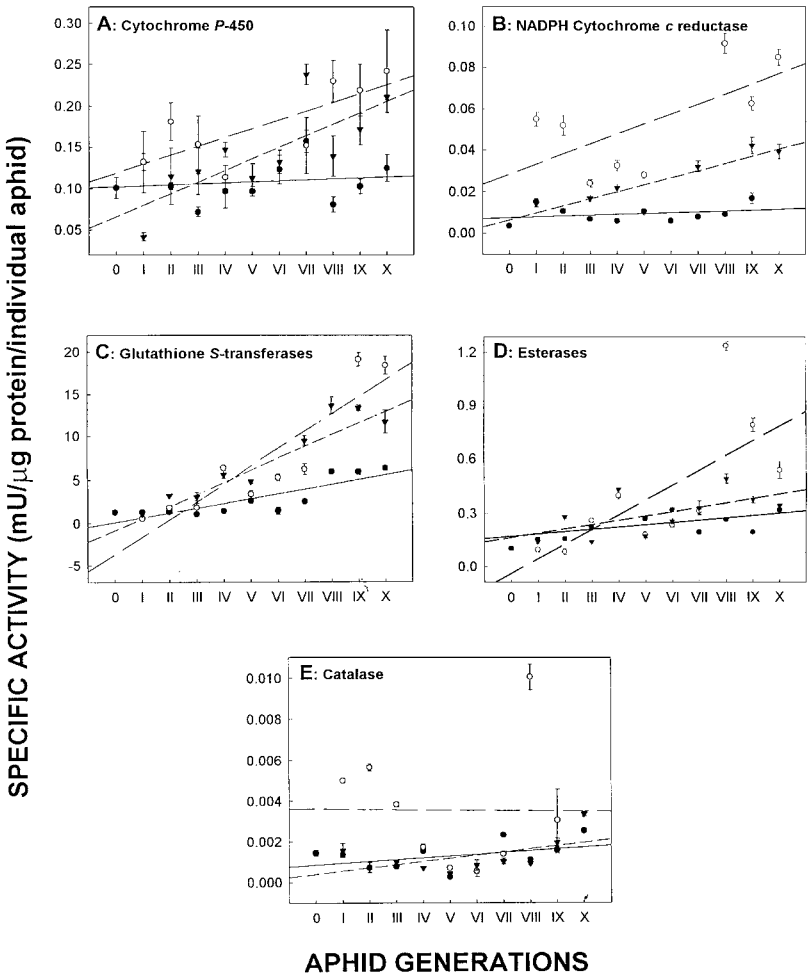

FIG. 2. Effect of two wheat cultivars and oat on the specific activity per aphid individual (mean ± SE) of five enzymes along 10 generations of *S. avenae*. Oat: •, solid line; wheat cultivar Huayún: ○, long-dashed line; and wheat cultivar Chagual: ▼, short-dashed line. The slopes of the lines and the ANCOVA comparisons between the slopes are shown in Table 1. Values for generation 0 correspond to the specific activity assayed in aphids from the stock culture on oat.

and catalase, respectively. The overall trend of constant activity for the enzymes in aphids from oat makes oat a reliable control for the purpose of the present study.

TABLE 1. EFFECT OF TWO WHEAT CULTIVARS AND OAT ON SPECIFIC ACTIVITY PER APHID INDIVIDUAL (MEAN ± SE) OF FIVE ENZYMES ALONG TEN GENERATIONS OF *Sitobion avenae*

	Slopes of plots "specific activity per aphid individual vs. generation"			*P* values for ANCOVA comparisons between slopes*		
Enzyme	Oat	Huayún	Chagual	Oat–Huayún	Oat–Chagual	Huayún–Chagual
Cy*P*450	0.001	0.012	0.014	0.05	<0.01	0.656
NADPH cyt red	0.104	4.87	4.01	<0.05	<0.05	<0.005
GSTs	0.62	2.04	1.38	<0.001	<0.005	<0.005
Esterases	0.008	0.082	0.024	<0.05	0.064	<0.001
Catalase	0.127	−0.013	0.155	0.977	0.157	<0.005

*$P > 0.05$: differences between slopes are not significant.

The mixed function oxidase complex has been proposed as a general-purpose detoxification system. While Cy*P*450 oxidizes a broad range of lipophilic xenobiotic molecules by converting them into more polar substances (Ahmad et al., 1986; Yu, 1987; Leszczynski et al., 1994), NADPH cytochrome *c* reductase reduces the oxygen molecule incorporated to the Cy*P*450 complex during oxidation of xenobiotics (Amar-Costesec et al., 1974; Ahmad et al., 1986). Cy*P*450 is particularly important in polyphagous herbivores that encounter a wide range of plant allelochemicals (Yu, 1987). Consistent with this concept is the inducibility of these enzymes by a number of plant secondary compounds (Brattsen, 1977; Yu, 1986). The induction of Cy*P*450 in insects by Hx has been described. Thus, about a twofold increase in the activity of these enzymes was observed in *Ostrinia furnacalis* (Guenée) (Lepidoptera) fed on artificial diets containing DIMBOA (Yan et al., 1995). Moreover, in vitro experiments showed an increase in Cy*P*450 activity in homogenates of the aphid *Rhopalosiphum padi* (L.) when fed with DIMBOA-containing diets with respect to those without DIMBOA (S. Mukanganyama, personal communication). In the present work, a significant effect on the rate of change of activity of Cy*P*450 was observed in aphids from both wheat cultivars compared with oat, along ten generations (Table 1, Figure 2A), but no differences were found between wheat cultivars (Table 1). The increase of activity observed in aphids from both wheat cultivars suggests the occurrence of oxidation reactions involving secondary metabolites present in wheat but not in oat, such as Hx, and the concomittant induction of the MFO complex. The mechanism of metabolization of Hx is not known, however, it may be speculated that the MFO complex may hydroxylate some of the unsubstituted aromatic carbon atoms, leading to phenols, which, on account of their higher polarity, would be more readily excretable. Although examples of Cy*P*450 metabolizing the inducer allelochemical itself are scarce (Nitao; 1989),

the induction of activity of this detoxification pathway by Hx supports the hypothesis that these enzymes are ecologically significant in host-plant selection by insects (Nitao, 1989), particularly in aphids.

Recently, it was shown that NADPH cyt red activity was not significantly altered in aphids fed with DIMBOA-containing artificial diets with respect to control diet (Figueroa et al., 1999). Significant effects of both wheat cultivars as compared to oat were found on rates of change of enzymatic activity (Table 1, Figure 2B). Significant differences were also found between the slopes of wheat cultivars (Table 1). The induction of activity observed in aphids from both wheat cultivars is consistent with the effects found on Cy*P*450, and supports the role of the MFO system in the detoxification of Hx.

Glutathione *S*-transferases conjugate reduced glutathione (GSH) to electrophilic substrates, resulting in less toxic metabolites that can be stored or excreted (Yu, 1982; Wadleigh and Yu, 1988). GSTs can transform a variety of plant toxins including coumarins, indoles, flavonoids, isothiocyanates, and monoterpenes (Ahmad et al., 1996). Since their activity can be induced by various allelochemicals, including their own substrates, commonly found in insect food plants, it is likely that GSTs play a significant role in the detoxification of plant toxins in certain insects (Yu, 1992, 1996; Wadleigh and Yu, 1988). An induction of the activity of GSTs in *S. avenae* has been correlated positively with the concentration of Hx in the wheat cultivars where the aphids fed (Leszczynski and Dixon, 1990; Leszczynski et al., 1994); an increase in GSTs activity also has been reported in *O. furnacalis* fed on artificial diets containing DIMBOA (Yan et al., 1995). However, a decrease in GSTs activity has been shown when the aphid *R. padi* was allowed to feed on artificial diets with increasing concentrations of DIMBOA (S. Mukanganyama, personal communication). In the present work, an induction of specific activity was observed after *S. avenae* aphids fed on wheat cultivars with different Hx concentrations (Figure 2C). Comparison of slopes showed significant effects on activity induction in both wheat cultivars compared to oat (Table 1, Figure 2C). Significant effects were also found between wheat cultivars (Table 1). GSTs could metabolize DIMBOA in two nonmutually exclusive ways: glutathione could be conjugated with DIMBOA through the electrophilic carbonyl groups of the open chain tautomer (Figure 1) (Pérez and Niemeyer, 1989), or GSTs could use as substrate the products of MFO oxidation of Hx, whose nitrogen atom would be rendered more electrophilic by the phenolic hydroxyl groups. The present results suggest that an induction of GSTs by exposure of aphids to wheat cultivars that differ in Hx concentration may constitute a response of the detoxification metabolism of *S. avenae* against these important wheat secondary metabolites.

Esterases hydrolyze esters and amides, converting them into more polar compounds (Ahmad et al., 1986). Induction of esterases has been related with increased resistance to allelochemical toxicity (Ahmad et al., 1986). Increased

carboxylesterase activity as well as amplification of esterase genes is involved in the resistance to organophosphorus insecticides of the aphid *Myzus persicae* (Field et al., 1988; Field and Devonshire, 1992). Comparison of slopes in Figure 2D showed significant effects of wheat cv. Huayún on the rate of change of activity compared to oat and between the slopes corresponding to the two wheat cultivars (Table 1). Inactivation of acetylcholinesterase activity of the lepidopteran *O. furnacalis* and the aphid *R. padi*, has been observed when the insects fed on artificial diets containing DIMBOA (Cuevas and Niemeyer, 1993; Yan et al., 1995). This latter observation does not contradict the present results since acetylcholinesterases represent only part of the esterases present in an aphid.

Catalase is an enzyme present in the matrix of peroxisomes and in the cytosol. It decomposes hydrogen peroxide into water and molecular oxygen (Mannaerts and Van Veldhoven, 1993). Since oxidation reactions performed by detoxifying enzymes such as Cy*P*450 could produce reactive oxygen species, including the superoxide anion free radical and hydrogen peroxide (Ahmad et al., 1986), catalase can play an important indirect role in detoxification reactions. The specific activity found in this work in homogenates of aphids from oat (0.724 ± 0.066 mU/mg protein) is comparable to that recently reported for *S. avenae* aphids that fed on artificial diets lacking Hx (0.64 ± 0.064 mU/mg protein) (Figueroa et al., 1999). The lack of significant effects of wheat cultivars on rates of change of catalase activity (Table 1, Figure 2E) contrasts with the results of Figueroa et al. (1999) in which catalase activity was increased in *S. avenae* fed with DIMBOA-containing artificial diets with respect to control diets without DIMBOA. This may indicate that reactive oxygen species generated by the uncoupling of product formation and reducing equivalents from the MFO system may be neutralized by factors present in wheat phloem vessels that are absent from the artificial diets employed in the experiments of Figueroa et al. (1999). Alternatively, the metabolization of Hx may not be related with the production of reactive oxygen species.

Induction of enzyme activity was shown to be greater in the low-Hx wheat cultivar than in the high-Hx cultivar for NADPH cytochrome *c* reductase, glutathione *S*-transferases, and esterases. A plausible explanation of this finding is that the feeding deterrent effect of Hx limited the ingestion of Hx when they occurred at high concentrations in the plant. This effect has been shown to occur in the aphid *Metopolophium dirhodum* Walker. When bodies of aphids fed on wheat cultivars with different Hx levels or diets containing different concentrations of DIMBOA were analyzed, a maximum in the concentration of Hx in the aphids was found at intermediate Hx levels in the plants or in the diets (Niemeyer et al., 1989). The plant Hx concentration producing maximal accumulation in aphids is similar to that in cv. Huayún, and that producing minimal accumulation in aphids similar to that in Chagual (Niemeyer et al., 1989). Furthermore, aphids from cultivar Chagual were observed to be smaller than those from

cultivar Huayún in generations IV through X (Loayza-Muro, personal observation).

Enzymatic detoxification plays an important role in the chemical interaction between insects and their host plants, mediating insect adaptation to plant allelochemicals (Lindroth, 1989). Detoxification mechanisms in herbivorous insects are closely tuned to the specific chemistry of their hosts (Nitao, 1989), and the degree of induction of the detoxification enzymes is frequently influenced by the concentration of the allelochemicals in the plant (Wadleigh and Yu, 1988). Our findings show an increase in the activity of four enzymatic systems of aphids after their development for 10 generations on two wheat cultivars containing Hx relative to oat, lacking Hx. These results suggest the participation of this allelochemical as an inducer agent, although the apparent effect of the plant may result from the stimulatory, or synergistic, action of other allelochemicals present in cereals. For example, phenolic compounds, which are also related to resistance against aphids, are known to induce detoxifying enzymes, such as glutathione *S*-transferases (Leszczynski and Dixon, 1992).

Studies on detoxification metabolism in aphids have been performed on artificial diets or host plants for time periods no longer than one generation (Leszczynski and Dixon, 1992; Leszczynski et al., 1994; Figueroa et al., 1999). The overall increase of detoxification activity observed here through 10 generations suggests a probable mechanism of acquiring resistance to Hx that would not have been observed had the experiments been restricted to a single aphid generation. Thus, the selection of those clones showing higher inducibility of their detoxification metabolism would account for an overall steady build-up of resistance, which in turn should lead to an increase in aphid performance of wheat plant containing Hx throughout a season.

The observed effects of enzymatic activity of the cereal on which aphids fed may arise from a direct interaction between the enzyme and plant components such as Hx or from the de novo synthesis or inhibition of the synthesis of the enzyme (Yu, 1986). Biochemical studies exploring the routes followed by Hx inside aphids are needed to clearly understand the mechanisms through which this allelochemical affects, and is affected by, the enzymatic detoxification systems. In addition, studies addressing the consequences on aphid performance and fitness of such phenomena will reveal the extent to which these metabolic pathways enable aphids to adapt to the defense metabolites present in their host plants.

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